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Characterization of Site-Directed Mutants in the *lac* Permease of *Escherichia coli*. 2. Glutamate-325 Replacements

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ABSTRACT: *lac* permease with Ala in place of Glu325 was solubilized from the membrane, purified, and reconstituted into proteoliposomes. The reconstituted molecule is completely unable to catalyze lactose/H⁺ symport but catalyzes exchange and counterflow at least as well as wild-type permease. In addition, Ala325 permease catalyzes downhill lactose influx without concomitant H⁺ translocation and binds *p*-nitrophenyl α -D-galactopyranoside with a *K_D* only slightly higher than that of wild-type permease. Studies with right-side-out membrane vesicles demonstrate that replacement of Glu325 with Gln, His, Val, Cys, or Trp results in behavior similar to that observed with Ala in place of Glu325. On the other hand, permease with Asp in place of Glu325 catalyzes lactose/H⁺ symport about 20% as well as wild-type permease. The results indicate that an acidic residue at position 325 is essential for lactose/H⁺ symport and that hydrogen bonding at this position is insufficient. Taken together with previous results and those presented in the following paper [Lee, J. A., Püttner, I. B., & Kaback, H. R. (1989) *Biochemistry* (third paper of three in this issue)], the findings are consistent with the idea that Arg302, His322, and Glu325 may be components of a H⁺ relay system that plays an important role in the coupled translocation of lactose and H⁺.

The preceding paper (Püttner et al., 1989) confirms and extends earlier observations (Padan et al., 1979, 1985; Patel et al., 1982; Garcia et al., 1982; Püttner et al., 1986) focusing on the importance of His322 in lactose/H⁺ symport by the

lac permease. Thus, evidence was presented indicating that His322 may be directly involved in lactose-coupled H⁺ translocation.

Although a high-resolution structure for *lac* permease is not available, recent studies (Costello et al., 1984, 1987; Li & Tooth, 1987) suggest the presence of a notch or groove within the molecule. Therefore, the number of amino acid residues directly involved in substrate and H⁺ translocation may be fewer than the number of residues required to span the full thickness of the membrane.

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On the basis of various algorithms for secondary structure, *lac* permease is proposed to consist of 12–14 transmembrane α -helical domains (Foster et al., 1983; Bieseler et al., 1985; Wright et al., 1985; Vogel et al., 1985) with His322 and Glu325 located in putative transmembrane helix X (Foster et al., 1983). General aspects of the models are supported by biochemical and biophysical studies [cf. Kaback (1983, 1986)]. Bieseler et al. (1985) have also provided evidence that His322 and Glu325 are located in an α -helical portion of the polypeptide.

When putative helix X (Foster et al., 1983) is subjected to molecular modeling (Kaback, 1987), Glu325 is on the same face of the helix as His322, and the carboxylate is about 1.5 Å from the imidazole. On these grounds, it seems likely that the two residues are ion-paired, and for this reason, Carrasco et al. (1986) replaced Glu325 with Ala by using site-directed mutagenesis. Permease altered in this fashion does not catalyze lactose/ H^+ symport, but the protein catalyzes exchange and counterflow as well as wild-type permease. On the basis of these observations, it was suggested that His322 and Glu325 may be components of a charge-relay system that is important in lactose-coupled H^+ translocation. Subsequently, it was shown that Arg302 (putative helix IX), which is also important for lactose/ H^+ symport, may be sufficiently close to His322 to form a hydrogen bond, thereby forming the third component in a so-called catalytic triad [Menick et al., 1987; cf. Kaback (1987, 1988) for reviews].

In view of the critical importance of Glu325 in lactose/ H^+ symport, permease with Ala at position 325 was solubilized from the membrane, purified, and reconstituted into proteoliposomes. As shown here, the purified, reconstituted mutant permease behaves in a fashion identical with that reported for intact cells and right-side-out (RSO)¹ membrane vesicles. In addition, other amino acid replacements were made for Glu325, and the properties of the altered permeases were studied in RSO membrane vesicles. Although permease with Asp325 is still able to catalyze lactose/ H^+ symport, permeases with Gln, His, Val, Cys, or Trp at position 325, like permease with Ala325, do not catalyze lactose/ H^+ symport but exhibit normal exchange and counterflow activity. In the last paper of the series (Lee et al., 1989), further evidence supporting the notion that His322 and Glu325 are ion-paired is presented.

EXPERIMENTAL PROCEDURES

Materials

All materials utilized were of reagent grade and obtained from commercial sources as described (Sarkar et al., 1986).

Methods

Bacterial Strains. The bacterial strains used have been described (Sarkar et al., 1985; Püttner et al., 1986, 1989; Menick et al., 1987).

Oligonucleotide Synthesis. Deoxyoligonucleotides were synthesized on an Applied Biosystems Model 380B DNA synthesizer and purified by polyacrylamide gel electrophoresis as described in the Applied Biosystems manual.

Site-Directed Mutagenesis. Oligonucleotide-directed, site-specific mutagenesis (Zoller & Smith, 1983) was performed essentially as described (Sarkar et al., 1986) with one of the following modifications to improve the frequency of mutant recovery: (i) For E325A² (Carrasco et al., 1986) and

E325D, template single-stranded (ss) DNA was isolated from phage grown in *Escherichia coli* BW313 (*dut-ung*⁻) as described by Kunkel (1985). Closed-circular heteroduplex DNA with the desired mutations was synthesized in vitro as described (Sarkar et al., 1986) and transfected into *E. coli* JM109 (*ung*⁺). Mutant phage were screened as described below. (ii) For each of the other mutants described, mismatch repair was minimized by transfecting the heteroduplex into the mutator strain *E. coli* BMH71-18 *mutL* (Kramer et al., 1984). Phage harboring a given mutation were identified initially by colony-blot hybridization with the appropriate ³²P-labeled mutagenic primer (Carter et al., 1984). Phage from positive colonies were plaque purified, and the mutations were verified by dideoxyoligonucleotide sequencing (Sanger et al., 1977; Sanger & Coulson, 1978) utilizing a synthetic primer complementary to a region of *lac Y* 50–150 bases downstream from codon 325. In addition, the entire *lac Y* gene encoding the E325A and E325D mutations was sequenced by using six synthetic primers complementary to appropriate regions of *lac Y*.

Double-stranded *lac Y* DNA from each mutant was restricted from M13mp19 replicative form (RF) DNA and ligated into the *Eco*RI site of pACYC184 DNA. The resulting plasmids (Table I) were used to transform *E. coli* T184 (*Z*⁻*Y*⁻) or HB101 (*Z*⁺*Y*⁻), as indicated.

Measurement of Lactose-Induced H^+ Translocation. Measurements of intravesicular pH were measured in proteoliposomes containing entrapped 8-hydroxy-1,3,6-pyrenetrissulfonate (pyranine) as described by Matsushita et al. (1984). Proteoliposome reconstituted with wild-type or E325A permease in 50 mM potassium phosphate (pH 7.5)/1 mM dithiothreitol were diluted 5-fold, and pyranine was added to a final concentration of 2.5 mM. The preparations were then sonicated briefly in order to equilibrate the intravesicular and extravesicular compartments. Pyranine-loaded proteoliposomes were diluted 100-fold with 10 mM potassium phosphate (pH 7.5), harvested by centrifugation for 1 h at 175000g_{max}, and resuspended in 10 mM potassium phosphate (pH 7.5) to a final concentration of 42 µg of protein/mL and 31.3 mg of phospholipid/mL. Valinomycin was then added to a final concentration of 10 µM. In order to monitor changes in intravesicular pH, proteoliposomes loaded with pyranine were diluted to 0.14 µg of protein/mL in 10 mM potassium phosphate (pH 7.5), and the fluorescence of the entrapped pyranine was measured in a Perkin-Elmer MPF-66 fluorescence spectrophotometer (excitation, 460 nm; emission, 520 nm).

Other methods are described in the preceding paper (Püttner et al., 1989).

RESULTS

Verification of Mutations by DNA Sequencing

The *lac Y* gene in each plasmid used was cloned initially from pGM21 into M13mp19 RF DNA, and ss phage DNA was isolated and used as a template for site-directed mutagenesis. Subsequently, ss phage DNA containing mutated *lac Y* was isolated and sequenced (Sanger et al., 1977; Sanger & Coulson, 1978) with appropriate primers complementary to regions of *lac Y* 50–150 bases downstream from codon 325. The sequence analyses summarized in Table I demonstrate

¹ Abbreviations: RSO, right side out; ss, single stranded; RF, replicative form; EMB, eosin-methylene blue; PMS, phenazine methosulfate; Dns⁶-Gal, 6-(*N*-dansylamino)hexyl β-D-thiogalactopyranoside; NPG, *p*-nitrophenyl α-D-galactopyranoside.

² Site-directed mutants are designated as follows: The one-letter amino acid code is used followed by a number indicating the position of the residue in wild-type *lac* permease. The sequence is followed by a second letter denoting the amino acid replacement of this position (e.g., E325A designates that Glu325 is replaced with Ala).

Table I: DNA Sequence Analyses of E325 Mutants in *lac Y*^a

plasmid	mutagenic primer	DNA sequence of codon 325	amino acid
pGM21		GAA	Glu
pE325A	5'-GTACTGCAAACATATGC-3'	GCA	Ala
pE325C	5'-GAACGGTACACAAAACATATG-3'	TGT	Cys
pE325D	5'-GGTACGTCAAACATATGC-3'	GAC	Asp
pE325H	5'-GAACGGTACATGAAACATATGCAGCGTTTT-3'	CAT	His
pE325Q	5'-GAACGGTACTTGAAACATATG-3'	CAA	Gln
pE325V	5'-GAACGGTACTACAAAACATATGC-3'	GTA	Val
pE325W	5'-CAGGAACGGTACCCAAAACATATGCAG-3'	TGG	Trp

^a Italics indicates nucleotide or amino acid replaced in mutants.

that the mutated *lac Y* genes contain changes in codon 325 such that Glu is replaced with Ala, Asp, Gln, His, Cys, Val, or Trp as indicated. In addition, the entire nucleotide sequence of *lac Y* encoding the E325A and E325D mutations was determined by using six synthetic sequencing primers. With the exception of the alterations given in Table I, the remainder of the *lac Y* sequence was identical with that reported by Büchel et al. (1980).

E325A Permease

Preliminary studies by Carrasco et al. (1986) with intact cells and RSO membrane vesicles suggest that Glu325 plays a critical role in the release of H⁺ from the permease during turnover. Thus, permease with E325A is defective in all translocation reactions that involve net H⁺ movement but catalyzes equilibrium exchange and counterflow normally. In view of the unique properties of the E325A permease, the molecule was solubilized from the membrane, purified, and reconstituted into proteoliposomes by previously described techniques [cf. Viitanen et al. (1986)].

Active Transport. As shown previously (Newman et al., 1981; Foster et al., 1982) and in Figure 1, proteoliposomes reconstituted with purified wild-type permease catalyze lactose accumulation in the presence of a potassium diffusion gradient ($[K^+]_{in} > [K^+]_{out}$) and the ionophore valinomycin. In marked contrast, proteoliposomes reconstituted with purified E325A permease exhibit essentially no lactose transport under identical conditions.

Downhill Lactose Influx and Lactose-Induced H⁺ Translocation. Like the phenotypes conferred by plasmids pH322R (Padan et al., 1985), pH322N, pH322Q, pH322K (Püttner et al., 1989), and pR302L (Menick et al., 1987), when the cryptic strain HB101 is transformed with pE325A and grown at high lactose concentrations, the cells appear as red colonies on eosin-methylene blue (EMB) indicator plates (Carrasco et al., 1986). Therefore, although E325A permease is unable to catalyze lactose/H⁺ symport, the polypeptide is apparently able to facilitate downhill lactose translocation at high concentrations of the disaccharide presumably without concomitant H⁺ translocation.

This contention receives strong support from direct measurements of lactose-facilitated diffusion and lactose-induced H⁺ translocation (Figure 2). When 10 mM lactose is added to a suspension of proteoliposomes reconstituted with purified wild-type permease or purified E325A permease in the presence of valinomycin and nigericin, the internal lactose concentration equilibrates with the external medium within 10–15 min, and the rate of equilibration is dramatically inhibited by addition of *p*-(chloromercuri)benzenesulfonate (*p*CMBS) (Figure 2A). Although not shown, equilibration occurs within 3 h in the presence of *p*CMBS with both preparations.

In the corollary experiment, lactose-induced internal pH changes were monitored in the same proteoliposomes containing entrapped pyranine, an impermeant pH-sensitive fluorophore (Clement & Gould, 1981; Matsushita et al., 1984)

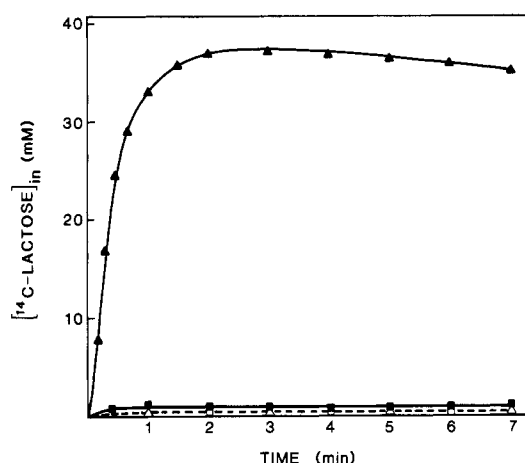


FIGURE 1: Membrane potential ($\Delta\Psi$) driven lactose transport in proteoliposomes reconstituted with purified wild-type (\blacktriangle , \triangle) or E325A permease (\blacksquare , \square). *lac* permease from *E. coli* T206 or E325A was solubilized, purified, reconstituted into proteoliposomes, and assayed for $\Delta\Psi$ -driven lactose transport as described [cf. Viitanen et al. (1986)]. The suspension contained 50 μ g of protein/mL and 37.5 mg of *E. coli* phospholipid/mL in 50 mM potassium phosphate (pH 7.5)/1 mM dithiothreitol. Valinomycin was added to 20 μ M final concentration, and aliquots (1 μ L) were diluted 200-fold into 50 mM sodium phosphate (pH 7.5; closed symbols) or 50 mM potassium phosphate (pH 7.5; open symbols) containing 0.3 mM [14 C]lactose (19.4 mCi/mmol). At given times, reactions were terminated, and the samples were assayed by rapid filtration and liquid scintillation spectrometry.

(Figure 2B). When added to proteoliposomes containing wild-type permease, lactose causes a transient decrease in fluorescence, indicating that the internal space becomes acidic as the result of lactose/H⁺ symport. The decrease in fluorescence reaches a minimum in about 5–6 min and is rapidly and completely reversed upon addition of nigericin. In contradistinction, proteoliposomes containing E325A permease do not exhibit any change in fluorescence upon addition of lactose. Thus, E325A permease catalyzes downhill influx of lactose, but the process does not occur in symport with H⁺. Although not shown, similar results were obtained when changes in external pH were measured directly with a pH electrode [cf. Püttner et al. (1989)].

Efflux, Exchange, and Counterflow. When proteoliposomes reconstituted with purified wild-type permease are equilibrated with 10 mM [14 C]lactose, treated with valinomycin, and diluted into medium devoid of lactose (Figure 3A) or into medium containing 10 mM lactose (Figure 3B), efflux and equilibrium exchange occur at rates approximating those described previously [i.e., $t_{1/2} \sim 1$ min and 10 s, respectively; cf. Garcia et al. (1983), Viitanen et al. (1983), and Püttner et al. (1989)]. In contrast, proteoliposomes reconstituted with E325A permease catalyze efflux at a rate that is only marginally greater than that observed in the presence of *p*CMBS, and the phenomenon is totally unaffected by increasing ambient pH to 9.5 (Figure 3A). As shown previously (Viitanen

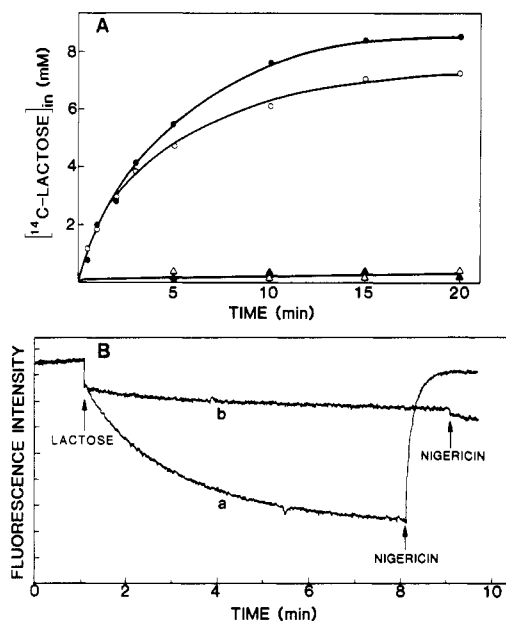


FIGURE 2: (A) Facilitated diffusion of lactose in proteoliposomes reconstituted with purified permeases. Proteoliposomes reconstituted with purified *lac* permease from *E. coli* T206 (●) or A325 (○) were concentrated to approximately 250 $\mu\text{g}/\text{mL}$ in 50 mM potassium phosphate (pH 7.5)/1 mM dithiothreitol, and facilitated diffusion was assayed as described [cf. Viitanen et al. (1986)]. Valinomycin and nigericin were added to final concentrations of 20 and 2 μM , respectively, and aliquots (1 μL) were diluted 100-fold into 50 mM potassium phosphate (pH 7.5) containing 10 mM $[^{14}\text{C}]\text{lactose}$ (7 mCi/mmol) at 25 $^{\circ}\text{C}$. At indicated times, reactions were terminated by rapid dilution with 4 mL of ice-cold 50 mM potassium phosphate (pH 7.5), and the samples were filtered immediately through Millipore GSTF filters (0.2 μm) and washed twice with the same volume of cold buffer. The data were corrected for zero-time control values obtained by adding an aliquot (1 μL) of proteoliposomes to reaction mixtures that had already been diluted with 4 mL of cold buffer, followed by filtration and washing. (▲ and △) Proteoliposomes reconstituted with wild-type and A325 permease, respectively, after removal of dithiothreitol and incubation with 2.5 mM pCMBS. Although not shown, after 3 h, the samples achieved the same internal concentration of lactose as the samples incubated in the absence of pCMBS. (B) Lactose-induced H^{+} translocation. Proteoliposomes reconstituted with wild-type (a) or E325A permease (b) were loaded with pyranine as described under Experimental Procedures, and changes in intravesicular pH were measured by monitoring the fluorescence of entrapped pyranine (excitation, 460 nm; emission, 520 nm). At indicated times, lactose or nigericin was added to the cuvette to final concentrations of 10 mM or 8.3 nM, respectively. The nigericin-sensitive fluorescence change observed with proteoliposomes containing wild-type permease (trace a) was 15% of the initial pyranine fluorescence.

et al., 1983), the rate of efflux in proteoliposomes reconstituted with wild-type permease is increased approximately 5-fold over this pH range. Remarkably, however, the rate of exchange in proteoliposomes with E325A permease is identical with that of proteoliposomes with wild-type permease within experimental error (Figure 3B). Furthermore, proteoliposomes with E325A permease catalyze counterflow at essentially the same rate and to the same extent as proteoliposomes with wild-type permease, but the internal concentration of $[^{14}\text{C}]\text{lactose}$ in E325A proteoliposomes is maintained at high levels for a prolonged period relative to proteoliposomes with wild-type permease (Figure 4A).

Interestingly, when the external $[^{14}\text{C}]\text{lactose}$ concentration is decreased to about half the K_m for entrance counterflow, the maximum level achieved during the overshoot in E325A proteoliposomes is about 3-fold greater than that in proteoliposomes containing wild-type permease (Figure 4B). The effect is significantly more marked than observed previously

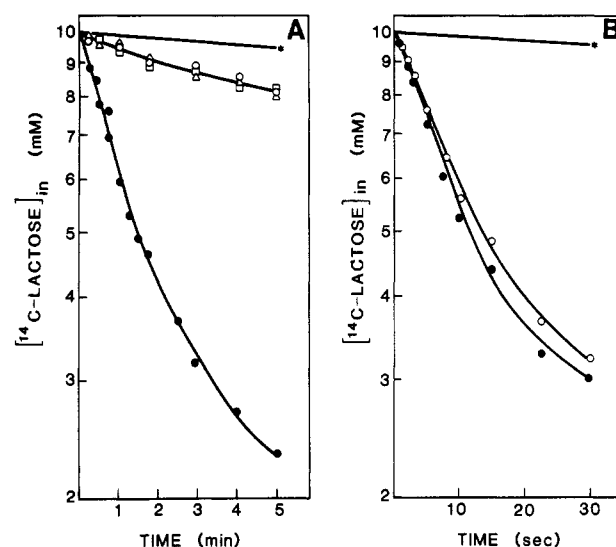


FIGURE 3: Lactose efflux (A) and equilibrium exchange (B). (A) Proteoliposomes containing purified *lac* permease from *E. coli* T206 (●) or E325A (○) were resuspended to 50 μg of protein/mL in 50 mM potassium phosphate/1 mM dithiothreitol at given pH values. A small aliquot of $[^{14}\text{C}]\text{lactose}$ (10 mCi/mmol) was added to each suspension to a final concentration of 10 mM, and valinomycin was added to a final concentration of 20 μM . After equilibration at room temperature for 1 h, aliquots (1 μL) were rapidly diluted into 200 μL of 50 mM potassium phosphate at the appropriate pH at 25 $^{\circ}\text{C}$. At indicated times, samples were diluted and filtered as described [cf. Viitanen et al. (1986)]. The experiments were conducted at pH 7.5 (○), 8.5 (△), or 9.5 (□). Data are presented as the concentration of lactose (mM) retained within the proteoliposomes as a function of time. (*) Identical experiments were performed with proteoliposomes incubated with pCMBS at a final concentration of 2.5 mM, as described in Figure 2. (B) Experiments were performed as described in (A), except that 10 mM unlabeled lactose was included in the medium into which the proteoliposomes were diluted.

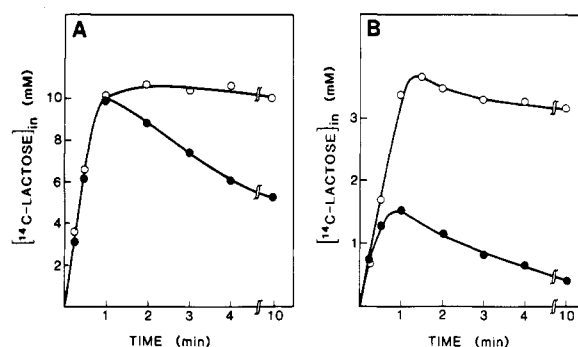


FIGURE 4: Lactose counterflow at saturating (A) and subsaturating (B) external lactose concentrations. Proteoliposomes reconstituted with purified *lac* permease from *E. coli* T206 (●) or E325A (○) were equilibrated with 10 mM lactose in the presence of 20 μM valinomycin. Counterflow was then assayed at given times at 25 $^{\circ}\text{C}$ by diluting an aliquot (1 μL) into 200 μL of 50 mM potassium phosphate (pH 7.5) containing 1.6 mM $[^{14}\text{C}]\text{lactose}$ (10 mCi/mmol) as described by Viitanen et al. (1986). (B) Experiments were conducted as described in (A), except that the dilution medium contained 0.25 mM $[^{14}\text{C}]\text{lactose}$ (40 mCi/mmol).

with deuterium in place of protium (Viitanen et al., 1983), in the presence of monoclonal antibody 4B1 (Carrasco et al., 1984), or in RSO vesicles from the uncoupled mutant *E. coli* ML 308-22 (Herzlinger et al., 1985) and is consistent with the notion that E325A permease is unable to release H^{+} from the permease during turnover [cf. Carrasco et al. (1986) and Kaback (1983, 1986)].

p-Nitrophenyl α -D-Galactopyranoside (NPG) Binding. As shown previously, wild-type *lac* permease binds NPG stoichiometrically with a K_D of about 20 μM (Rudnick et al., 1976; Herzlinger et al., 1985), while permeases with R302L (D. R.

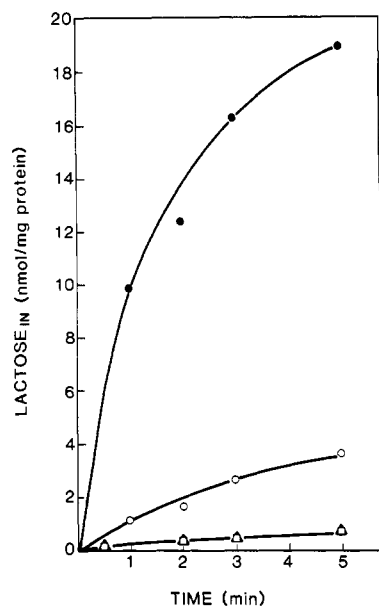


FIGURE 5: Time course of lactose transport in RSO vesicles from *E. coli* T206 (●), E325D (○), E325A (□), E325C (□), E325H (□), E325Q (□), E325V (□), E325W (□), or T184 (Δ). RSO membrane vesicles were prepared and assayed for lactose transport in the presence and absence of reduced PMS as described (Kaback, 1971, 1974). Transport in the presence or absence of electron donors was indistinguishable from that shown for E325C, E325H, E325Q, E325V, or E325W (□) or for T184 (Δ).

Menick, L. Patel, and H. R. Kaback, unpublished information) or H322R (Püttner et al., 1989) appear to bind ligand with markedly decreased affinity. Although not shown, Scatchard analyses of NPG binding data obtained with RSO membrane vesicles containing wild-type permease yield a K_D value of 35 μ M. Interestingly, the K_D observed with E325A vesicles is about 70 μ M, a value very significantly lower than that observed with either the R302L or H322R permease. Thus, the affinity of the permease for NPG appears to be relatively unaffected by replacement of Glu325 with Ala.

Other Amino Acid Replacements for Glu325

In order to more fully elucidate the role of Glu325 in lactose/ H^+ symport, codon 325 of *lac Y* was modified by site-directed mutagenesis so that Glu325 in the permease was replaced with Asp, Gln, His, Val, Cys, or Trp (Table I). Subsequently, the effects of the replacements on permease activity were studied qualitatively by plating *E. coli* HB101 transformed with the appropriate plasmid on EMB/lactose and more quantitatively by assaying various translocation reactions in RSO membrane vesicles.

Colony Morphology on EMB/Lactose. *E. coli* HB101 (Z^+Y^-) transformed with pE325D grows as dark red colonies on EMB containing 25 mM lactose. That is, the cells exhibit a phenotype indistinguishable from that of cells that express wild-type permease. HB101 transformed with each of the other plasmids also appear as dark red colonies, indicating that they too are able to metabolize lactose effectively at high concentrations.

Active Transport. In the absence of exogenous electron donors, RSO membrane vesicles containing wild-type permease or each of the mutated permeases described transport lactose at negligible initial rates and to minimal steady-state levels of accumulation (Figure 5). On addition of ascorbate and phenazine methosulfate (PMS), the initial rate of transport in vesicles containing wild-type permease increases dramatically, and the vesicles achieve a steady-state level of accumulation by about 5 min. In contrast, vesicles containing

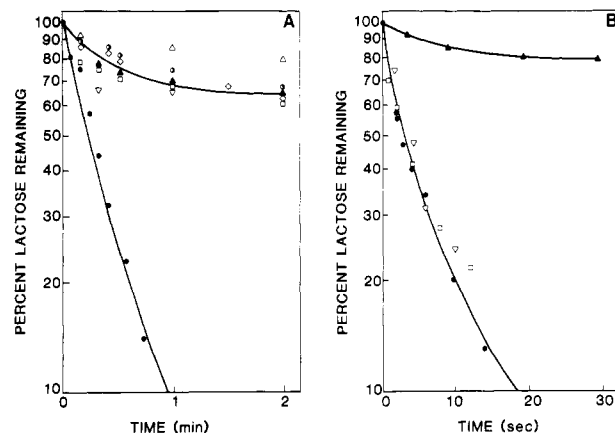


FIGURE 6: Lactose efflux (A) and exchange (B) in RSO membrane vesicles from *E. coli* T206 (●), E325C (○), E325H (◇), E325Q (□), E325V (Δ), and E325W (▽). Membrane vesicles were equilibrated with 10 mM [^{14}C]lactose (6 mCi/mmol), and aliquots were rapidly diluted into media devoid of lactose (A) or media containing equimolar concentrations of unlabeled lactose (B). At the times indicated, the reactions were terminated with 3 mL of 0.1 M potassium phosphate (pH 5.5)/0.1 M lithium chloride/20 mM $HgCl_2$, and the samples were immediately filtered as described (Kaczorowski & Kaback, 1979). Similar experiments were performed in the presence of 1 mM pCMBS (▲). Although not shown, similar results were obtained for equilibrium exchange with E325C, E325H, and E325V vesicles.

E325D permease transport lactose at about 20% of the rate of wild-type vesicles, and the steady-state level of accumulation is also reduced to a similar extent. Furthermore, no effect is observed after addition of PMS/ascorbate to vesicles containing permease with Ala, Gln, His, Cys, Val, or Trp in place of Glu325. Significantly, each vesicle preparation exhibits an amount of permease comparable to that observed in vesicles containing wild-type permease, as judged by immunoblot analyses [cf. Herzlinger et al. (1985)], and each preparation catalyzes respiration-driven proline transport normally (data not shown). Therefore, it is apparent that *lac* permease with Asp in place of Glu325 retains partial activity, while permease with each of the other amino acid replacements at position 325 is inactive.

Efflux, Exchange, and Counterflow. As shown with E325A vesicles (Carrasco et al., 1986) and with proteoliposomes containing purified E325A permease (Figure 3), RSO vesicles containing permease with Gln, Val, His, Cys, or Trp in place of Glu325 catalyze efflux at markedly defective rates (Figure 6A). In addition, it is noteworthy that the rate of efflux in vesicles containing E325C permease is not increased significantly when ambient pH is increased to pH 9.5 (data not shown). In contrast, the rate of equilibrium exchange catalyzed by each *lac* permease mutant is indistinguishable from the rate catalyzed by wild-type permease (Figure 6B). Moreover, E325A, E325Q, E325H, E325C, E325V, and E325W vesicles catalyze counterflow at similar rates and to similar extents as observed in T206 vesicles, but the increase in Dns⁶-Gal fluorescence is maintained for longer periods of time (Figure 7). Finally, as observed with purified, reconstituted E325A permease (Figure 4B), the mutant *lac* permeases catalyze lactose/Dns⁶-Gal counterflow more effectively than the wild type when the external concentration of Dns⁶-Gal is decreased to below the apparent K_m .

DISCUSSION

Use of site-directed mutagenesis has focused attention on specific amino acid residues that clearly play an important role in the mechanism of lactose/ H^+ symport [cf. Kaback (1987a,b, 1988) for reviews]. Thus, Arg302 (putative helix IX), His322

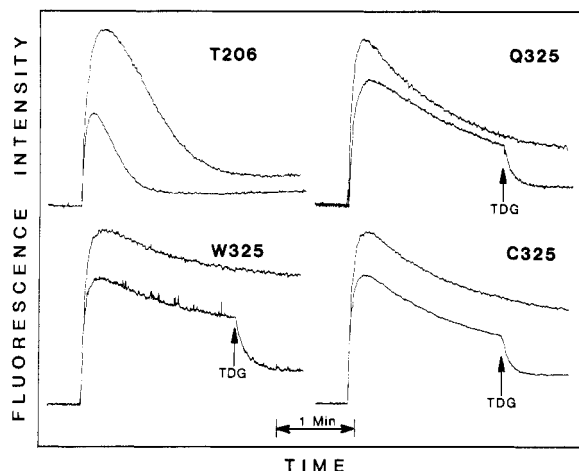


FIGURE 7: Dns⁶-Gal/lactose counterflow in RSO membrane vesicles from T206, E325W, E325Q, and E325C. Aliquots (4 μ L) of membrane vesicles equilibrated with 20 mM lactose in the presence of 2 μ M nigericin and 20 μ M valinomycin were diluted into 2 mL of 0.1 mM potassium phosphate (pH 6.6)/10 mM magnesium sulfate containing 20 μ M (upper curves, saturating) or 5 μ M (lower curves, subsaturating) Dns⁶-Gal (Schuldiner et al., 1975). Fluorescence emission was recorded continuously at 500 nm (excitation, 340 nm) in a Perkin-Elmer MPF-66 fluorescence spectrophotometer. Similar traces were recorded for RSO membrane vesicles from E325H, E325Q, and E325V (data not shown). Arrows designate addition of β -D-galactopyranosyl 1-thio- β -D-galactopyranoside (TDG) to a final concentration of 20 mM.

(putative helix X), and Glu325 (putative helix X) are critical for lactose/H⁺ symport, while certain other residues play no apparent role in the transport mechanism. Furthermore, evidence has been presented that is consistent with the idea that Arg302, His322, and Glu325 may be sufficiently close to form a hydrogen-bond network that is directly involved in H⁺ translocation.

The behavior of permease mutants specifically altered in Arg302, His322, or Glu325 is easily rationalized by the kinetic scheme presented in Figure 8. By the mechanism shown, efflux down a concentration gradient consists of a minimum of five steps: (1) binding of substrate and H⁺ to the permease on the inner surface (order unspecified); (2) translocation of the ternary complex to the outer surface; (3) release of substrate; (4) release of H⁺; (5) return of the unloaded permease to the inner surface. Alternatively, exchange and counterflow with external lactose at saturating concentrations involve steps 1–3 only (Kaczorowski & Kaback, 1979; Kaczorowski et al., 1979; Viitanen et al., 1983; Carrasco et al., 1984, 1986; Herzlinger et al., 1985). Since all steps in the mechanism that involve protonation or deprotonation are blocked in the R302L and H322R mutants and the primary effect of the H⁺ electrochemical gradient on the kinetics of lactose transport (i.e., a decrease in the apparent K_m for lactose; Kaczorowski et al., 1979; Viitanen et al., 1983) is not observed in these mutants, it seems likely that protonation of His322 is involved in step 1 in a manner that involves Arg302. In contrast, replacement of Glu325 with Ala results in permease that is defective in all steps that require net H⁺ translocation but catalyzes exchange and counterflow normally. For this reason, it was suggested (Carrasco et al., 1986) that Glu325 may be involved in step 4 of the mechanism (i.e., Glu325 may mediate loss of H⁺ from the permease).

The results presented here provide strong support for previous observations with E325A permease (Carrasco et al., 1986) and extend the conclusions significantly. Thus, it has been demonstrated that although purified, reconstituted permease with Ala in place of Glu325 is unable to catalyze

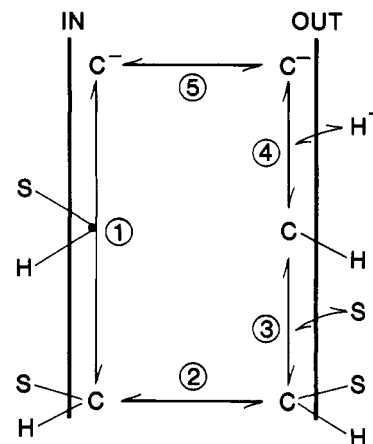


FIGURE 8: Schematic representation of reactions involved in lactose efflux, exchange, and counterflow. C represents *lac* permease. The order of substrate binding at the inner surface of membrane is not implied [from Kaczorowski and Kaback (1979)].

$\Delta\mu_{H^+}$ -driven active transport or efflux, both of which require net H⁺ translocation, E325A permease catalyzes equilibrium exchange and counterflow as well as wild-type permease. Furthermore, when counterflow is performed at external lactose concentrations below the apparent K_m , the altered permease functions better than the wild-type molecule which is consistent with the argument that E325A permease is blocked in step 4 of the kinetic mechanism (cf. Figure 8). Finally, it is apparent that E325A permease is completely uncoupled. That is, the purified, reconstituted permease catalyzes downhill lactose translocation at high concentrations of the disaccharide (i.e., facilitated diffusion), but the process occurs without H⁺ translocation.

Importantly, as evidenced by binding studies with NPG, E325A permease binds the high-affinity ligand with a K_D approximating that of wild-type permease. The finding is consistent with the observation that counterflow, a process that exhibits an apparent K_m similar to that observed for active transport, is intact in the mutant but is in marked contrast to findings with the R302L and H322R permeases which exhibit dramatically decreased affinities (D. R. Menick, L. Patel, and H. R. Kaback, unpublished information; Püttner et al., 1989). Therefore, in addition to postulating that His322 may be a component of the substrate binding site as well as an intermediate in H⁺ translocation (Püttner et al., 1989), it is tempting to speculate further that a protonated imidazole at position 322 may be required for high-affinity binding.

Although studies with mutant permeases containing other amino acid replacements for Glu325 were carried out with RSO membrane vesicles rather than proteoliposomes reconstituted with purified permease, the results are clear-cut. Permease with Asp in place of Glu325 catalyzes lactose/H⁺ symport about 20% as well as wild-type permease. Since the pK of Asp is lower than that of Glu and the amino acid side chain of Asp is 1.5–2.0 Å shorter than that of Glu, the result is not surprising. It is also noteworthy that E325D permease is probably partially uncoupled, although the stoichiometry between lactose and H⁺ translocation has not yet been measured directly. On the other hand, permeases with Gln, His, Cys, Val, or Trp behave in a manner indistinguishable from E325A permease. That is, permease with each of these amino acid replacements at position 325 is completely defective in lactose/H⁺ symport (i.e., active transport and efflux) but catalyzes equilibrium exchange and counterflow at rates approximating those of wild-type permease. Moreover, in each instance, the counterflow activity of the mutant is enhanced

relative to that of the wild type when the external substrate concentration is below the apparent K_m .

The observations have important implications on different levels: (1) Given the nature of the replacements and the ability of the modified permeases to catalyze exchange and counterflow, it seems unlikely that either the bulk or hydrophathy of the side chain at position 325 has a significant effect on substrate binding or translocation of the loaded permease. (2) Since permease with Asp325 retains partial ability to catalyze lactose/ H^+ symport, while permease with Gln325 catalyzes exchange and counterflow but is totally defective in all reactions involving net H^+ translocation, it is likely that an acidic residue at position 325 is required for lactose-coupled H^+ translocation and that hydrogen bonding is insufficient. (3) As discussed previously (Menick et al., 1987; Kaback, 1987), Arg302, His322, and Glu325 might function as components of a charge relay, in which H^+ would be expected to move physically from one residue to the other. Alternatively, however, His322 might be the only residue involved in H^+ translocation, and its pK_a may be modulated by the proximity of a positive charge in Arg302 and a negative charge in Glu325. In this regard, the behavior of the E325C mutant may be significant. As indicated, this mutant is unable to catalyze reactions involving net H^+ translocation even when the ambient pH is increased to pH 9.5, where Cys would be expected to exist as a thiolate anion. Although the observation tends to favor the charge-relay notion, it should be emphasized that the local pH within the protein may not be in equilibrium with the bulk phase and/or that the pK_a of a Cys within the permease may be perturbed (i.e., at an ambient pH of 9.5, Cys325 may not be negatively charged).

ACKNOWLEDGMENTS

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Registry No. Glu, 56-86-0; Ala, 56-41-7; Gln, 56-85-9; His, 71-00-1; Val, 72-18-4; Cys, 52-90-4; Trp, 73-22-3; Asp, 56-84-8; NPG, 7493-95-0; H^+ , 12408-02-5; lactose, 63-42-3; lactose permease, 9068-45-5.

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